

BID mediates selective killing of APC-deficient cells in intestinal tumor suppression by nonsteroidal antiinflammatory drugs

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Colorectal tumorigenesis is driven by genetic alterations in the *adenomatous polyposis coli* (APC) tumor suppressor pathway and effectively inhibited by nonsteroidal antiinflammatory drugs (NSAIDs). However, how NSAIDs prevent colorectal tumorigenesis has remained obscure. We found that the extrinsic apoptotic pathway and the BH3 interacting-domain death agonist (BID) are activated in adenomas from NSAID-treated patients. Loss of BID abolishes NSAID-mediated tumor suppression, survival benefit, and apoptosis in tumor-initiating stem cells in APC^{Min/+} mice. BID-mediated cross-talk between the extrinsic and intrinsic apoptotic pathways is responsible for selective killing of neoplastic cells by NSAIDs. We further demonstrate that NSAIDs induce death receptor signaling in both cancer and normal cells, but only activate BID in cells with APC deficiency and ensuing c-Myc activation. Our results suggest that NSAIDs suppress intestinal tumorigenesis through BID-mediated synthetic lethality triggered by death receptor signaling and gatekeeper mutations, and provide a rationale for developing more effective cancer prevention strategies and agents.

colorectal cancer | APC | chemoprevention | BID | apoptosis

Colorectal cancer (CRC) is the third leading cause of cancer-related death in the United States (1). However, treatment options for advanced colorectal cancer, including radiation, cytotoxic, and targeted therapies, are of limited effectiveness. Colorectal tumorigenesis is driven by genetic alterations in the APC tumor suppressor pathway through aberrant Wnt signaling, leading to β -catenin accumulation and activation of oncogenes such as c-Myc and CCND1 (2). One of the most promising strategies for reducing the morbidity and mortality of CRC is to inhibit tumorigenesis by using natural products or pharmacologic agents, such as nonsteroidal antiinflammatory drugs (NSAIDs) (3). The chemopreventive activities of NSAIDs, such as aspirin and sulindac, have been demonstrated in epidemiological studies (4), clinical trials (5, 6), and animal models (7). However, the critical cellular activities and molecular targets of NSAIDs in chemoprevention have remained elusive.

It is suggested that the antitumor effects of NSAIDs require selective killing of neoplastic cells through apoptosis (8), a major turnover mechanism of intestinal epithelial cells (9). Apoptotic death is regulated by the death receptor (DR or extrinsic) and mitochondrial (intrinsic) pathways (10). The DR pathway involves activation of death receptors such as DR4 and DR5, recruitment of FADD and procaspase 8, and depletion of pro-survival proteins such as c-FLIP, resulting in activation of the recruited caspase 8 and other caspases (11). The mitochondrial pathway is regulated by the Bcl-2 family proteins (12) and characterized by mitochondrial dysfunction, release of cytochrome c, and activation of caspase 9 (10). These two pathways cross talk under certain conditions through caspase-8-mediated cleavage of BID, a BH3-only Bcl-2 family member (13). The

activated and truncated BID (tBID) then engages the intrinsic pathway for efficient apoptosis induction (13). NSAIDs rely on the intrinsic pathway to kill CRC cells (14, 15). Nonetheless, it is still unclear whether apoptosis induction is essential for the antitumor activities of NSAIDs.

In this study, we found that NSAID treatment activates BID and the extrinsic apoptotic pathway in human intestinal adenomas. Loss of BID almost completely abolishes NSAID-mediated tumor suppression and killing of oncogenic intestinal stem cells in APC-deficient mice. BID is activated by a synthetic lethal interaction and mediates the effects of NSAIDs through cross-talk between the extrinsic and intrinsic pathways. Our results indicate that BID-dependent killing of tumor-initiating stem cells is critical for cancer prevention by NSAIDs.

Results

NSAIDs Activate Caspase 8 and BID in Human Colonic Adenomas. To determine the role of the extrinsic apoptotic pathway in NSAID-mediated tumor suppression, we used terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) and active caspase 8 staining to analyze advanced colonic adenomas from patients taking aspirin or other NSAIDs and control samples from patients without NSAID use (16). The number of TUNEL-positive cells in the adenomas of the NSAID-treated patients was 5.0-fold higher compared with those in the control group (Fig. 1A). Active caspase 8 positive cells were markedly increased in

Significance

Colorectal cancer is the third leading cause of cancer-related death in the United States, but treatment options for this disease are of limited effectiveness. Most human colorectal tumors begin with an inactivating mutation in the *adenomatous polyposis coli* (APC) gene. We demonstrate a mechanism by which nonsteroidal antiinflammatory drugs (NSAIDs) protect against colon cancer development by killing intestinal stem cells that have lost functional APC. NSAID treatment combines with APC loss-induced gene expression changes to selectively activate BID and induce apoptosis in these cells, while leaving normal cells unharmed. These results provide a rationale for developing more effective cancer prevention strategies and agents.

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The authors declare no conflict of interest.

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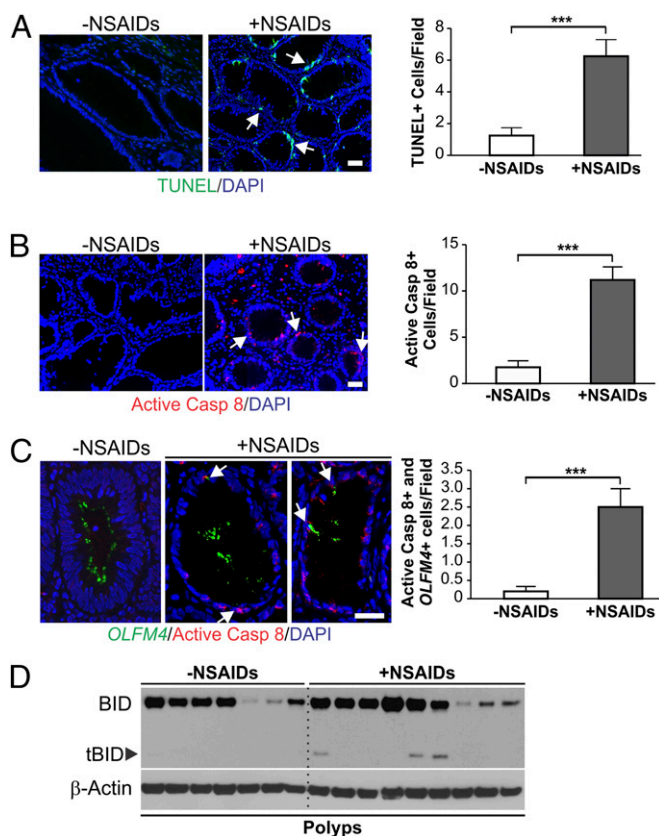


Fig. 1. NSAID treatment activates caspase 8 and BID in human advanced adenomas. (A–C) Advanced adenomas from nine patients taking NSAIDs and seven patients not taking NSAIDs were analyzed by TUNEL staining (green) (A), active caspase 8 (Casp 8) staining (red) (B), and OLFM4 (green) and active caspase 8 (red) double staining (C). (Left) Representative staining pictures. (Right) Mean numbers + SD of positive cells per field. (Scale bars: 25 μ m.) *** P < 0.001. Arrows indicate example positive cells. (D) BID expression in the adenomas was analyzed by Western blotting using homogenized tissue lysates. Each lane represents an individual patient.

the patients taking NSAIDs (Fig. 1B), and detected in 8 of 9 adenomas from NSAID users, but only in 1 of 7 from nonusers. Upon analyzing olfactomedin 4 (OLFM4) (Fig. 1C, Left), which marks human crypt base columnar cells (CBCs), representing active intestinal stem cells (ISCs) (17), we found active caspase 8/OLFM4 double-positive cells were significantly increased by more than 12-fold in the adenomas from NSAID takers compared with nonusers (Fig. 1C, Right). Most notably, BID cleavage was detected in several adenomas with high BID expression from NSAID users, but not in any adenomas from the patients not taking NSAIDs (Fig. 1D). These results suggest that NSAIDs kill oncogenic stem cells in human adenomas, and activation of the DR pathway and BID may contribute to this effect of NSAIDs.

BID Is Required for Tumor Suppression by NSAIDs in $APC^{Min/+}$ Mice.

To determine whether BID is required for the chemopreventive effects of NSAIDs, we crossed $APC^{Min/+}$ mice, a genetic model of intestinal adenomas (18), with BID KO mice (19) and generated age- and sex-matched cohorts of C57BL/6J $APC^{Min/+}$ mice with different BID genotypes. These mice were then fed an AIN-93G diet containing 0 (control) or 200 ppm sulindac, and analyzed for intestinal polyp formation. Sulindac treatment suppressed small intestinal adenoma formation in $BID^{+/+}/APC^{Min/+}$ mice by more than 80% compared with the untreated mice (Fig. 2A–C). Remarkably, this effect of sulindac was almost completely lost in $BID^{-/-}/APC^{Min/+}$ mice, in which the number of

small intestinal polyps was only slightly reduced (Fig. 2C). Sulindac was also less effective in the small intestine of BID heterozygous $APC^{Min/+}$ mice (Fig. 2C). Similar observations were made in the colon of $APC^{Min/+}$ mice (Fig. 2A–C). Indomethacin (10 ppm), also an NSAID with antitumor activity (15), was similarly BID-dependent in reducing small intestinal adenoma formation (Fig. S1). In untreated animals, loss of BID did not significantly affect the size and number of small intestinal polyps, but led to an increase in polyp number in the colon of $APC^{Min/+}$ mice (Fig. 2C).

We then compared the effects of prolonged sulindac treatment on the survival of $BID^{+/+}$ and $BID^{-/-}$ $APC^{Min/+}$ mice. In striking contrast to $BID^{+/+}/APC^{Min/+}$ mice ($n = 9$), which were alive after 70 wk of treatment, all of the $BID^{-/-}/APC^{Min/+}$ mice ($n = 11$) died before 35 wk, surviving only slightly longer than those on the control diet (~ 30 wk) (Fig. 2D). The difference between the $BID^{+/+}$ and $BID^{-/-}$ groups is highly significant (70 vs. 33 wk, $P = 0.0005$) (Fig. 2D). Together, these results demonstrate that BID plays an essential role in NSAID-mediated chemoprevention in $APC^{Min/+}$ mice.

BID Is Required for NSAID-Induced Killing of Intestinal Stem Cells in $APC^{Min/+}$ Mice.

We investigated how BID mediates the chemopreventive effects of NSAIDs in $APC^{Min/+}$ mice. We found that crypt cell apoptosis detected by TUNEL and active caspase 3 staining was significantly reduced in the small intestine and colon of sulindac- or indomethacin-treated $BID^{-/-}/APC^{Min/+}$ mice relative to $BID^{+/+}/APC^{Min/+}$ mice (Fig. 3A and B and Fig. S2A and B). In contrast, activation of caspase 8 by sulindac or indomethacin was not affected by the absence of BID (Fig. S2C). Sulindac was shown to preferentially kill ISCs in the crypt bottom of $APC^{Min/+}$ mice (16), which can be detected by markers such as *Lgr5* and *OLFM4* (20). Analysis of *Lgr5*-EGFP-marked mice revealed that the killing effect of sulindac and indomethacin on ISCs indicated by TUNEL/EGFP double-positive staining was reduced in the small intestine and colon of $BID^{-/-}/APC^{Min/+}$ mice relative to $BID^{+/+}/APC^{Min/+}$ mice (Fig. 3C and Fig. S2D). Using *OLFM4* RNA in situ hybridization to detect CBCs, we found the killing effect of sulindac and indomethacin on ISCs indicated by TUNEL/*OLFM4* double-positive staining was also reduced in the small intestine of $BID^{-/-}/APC^{Min/+}$ mice (Fig. S2E). Inhibition of cyclooxygenase 2 (COX 2) by NSAIDs has been implicated as a mechanism of NSAID-mediated chemoprevention (21). Sulindac and indomethacin treatment reduced plasma prostaglandin E2 metabolites (PGEM) by $\sim 50\%$ in $BID^{+/+}/APC^{Min/+}$, but these effects were intact in $BID^{-/-}/APC^{Min/+}$ mice (Fig. S2F), suggesting that BID functions downstream of COX 2 inhibition in suppressing tumorigenesis. Together, these results indicate that BID mediates the chemopreventive effects of NSAIDs through selective killing of APC -deficient ISCs.

BID Mediates NSAID-Induced Apoptosis Through the Mitochondrial Pathway.

To further delineate the functional role of BID in NSAID-mediated tumor suppression, we analyzed HCT116 colon cancer cells, which contain a β -catenin-activating mutation and can recapitulate the anticancer and apoptotic effects of NSAIDs in mice (14–16). We generated BID knockout (BID KO) HCT116 cells by using homologous recombination (Fig. S3 and Fig. 4A) and compared WT and BID KO cells for their responses to NSAIDs and other anticancer agents. Strikingly, apoptosis induced by sulindac and indomethacin, as determined by nuclear fragmentation and annexin V staining, was almost completely blocked in BID KO cells (Fig. 4B and Fig. S4A). BID deficiency abrogated NSAID-induced Bax multimerization (Fig. 4C), mitochondrial membrane depolarization (Fig. 4D), cytosolic release of cytochrome *c* and SMAC (Fig. 4E), and activation of caspases 9, 7, and 3 (Fig. 4F). Reconstituting BID expression in BID KO cells

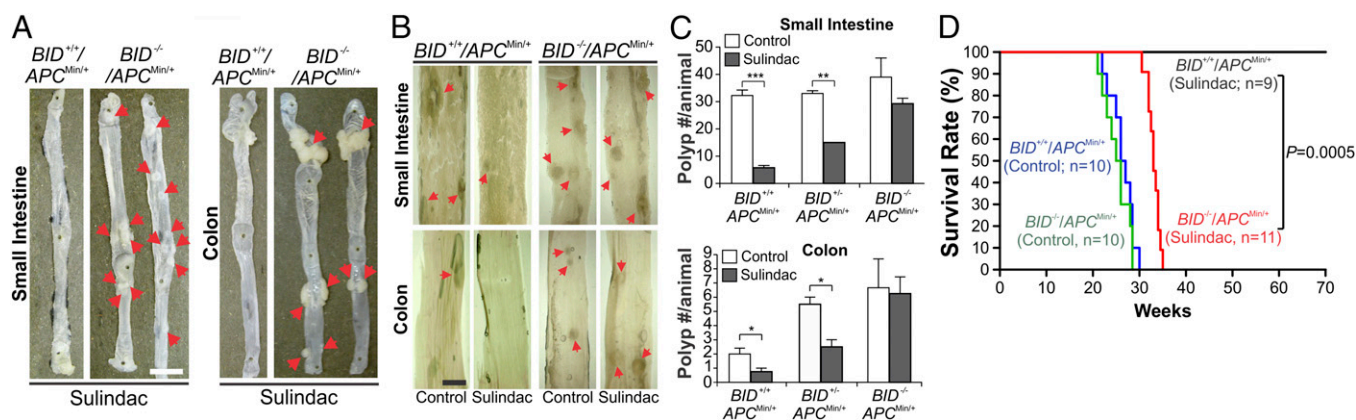


Fig. 2. BID is required for tumor suppression by NSAIDs in $APC^{Min/+}$ mice. Age- and sex-matched $APC^{Min/+}$ mice with different BID genotypes were fed control or NSAID-containing AIN93G diet. (A) Representative images of small intestine and colon from $BID^{+/+}$ and $BID^{-/-}$ $APC^{Min/+}$ mice treated with sulindac (200 ppm) for 4 mo, with arrows indicating visible macroadenomas. (B) Representative images of small intestine and colon of control mice and mice treated with sulindac (200 ppm) for 2 mo, with arrows indicating microscopic lesions. (C) Mean numbers + SD of small intestinal and colonic adenomas (≥ 0.5 mm in diameter) in $BID^{+/+}$, $BID^{-/-}$, and $BID^{-/-}$ $APC^{Min/+}$ mice treated \pm sulindac as in B. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ ($n = 4$ per group). (D) Kaplan–Meier survival curves of $BID^{+/+}$ and $BID^{-/-}$ $APC^{Min/+}$ mice treated \pm sulindac (200 ppm) for 70 wk. $BID^{+/+}$ vs. $BID^{-/-}$ $APC^{Min/+}$ mice, $P = 0.0005$ (log-rank test). (Scale bars: A, 1 cm; B, 2 mm.)

restored sulindac-induced apoptosis (Fig. S4B). In contrast to wild-type (WT) cells, the long-term survival of BID KO cells, as analyzed by colony formation assay, did not decrease following NSAID treatment (Fig. S4C).

NSAIDs also required BID to induce apoptosis in other CRC cell lines, including APC -mutant DLD1 and HT29 cells, and APC -WT RKO cells (Fig. S4D). Furthermore, BID-dependent apoptosis was induced by other NSAIDs, including sulindac sulfone, a sulindac derivative (22), and SC236, a COX 2-specific inhibitor structurally unrelated to sulindac and indomethacin (23) (Fig. S5 A–C). In contrast, BID deficiency did not affect apoptosis induced by staurosporine, camptothecin, or overexpression of the BH3-only protein PUMA, but attenuated apoptosis induced by tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) (Fig. S5D). These results demonstrate

a prominent and specific role of BID in mediating the anticancer and apoptotic effects of NSAIDs.

BID Is Activated by NSAIDs Through the Extrinsic Apoptotic Pathway.

We determined how NSAIDs activate BID to engage the mitochondrial pathway to kill colon cancer cells. tBID was observed in HCT116 cells treated with different NSAIDs (Fig. 5A and Fig. S5 A and B) and could be detected as early as 18 h after treatment (Fig. 5A). The short form of DR5, but not DR4, was induced by NSAIDs in WT and BID KO HCT116 cells (Fig. 5B and Fig. S6 A and B). The induction of DR5 by NSAIDs was independent of p53 (Fig. S6C), which can activate DR5 following DNA damage (24), and observed in different CRC cells (Fig. S6D). The short form of c-FLIP (FLIP_s), a negative regulator of caspase 8 activation, was markedly down-regulated before BID

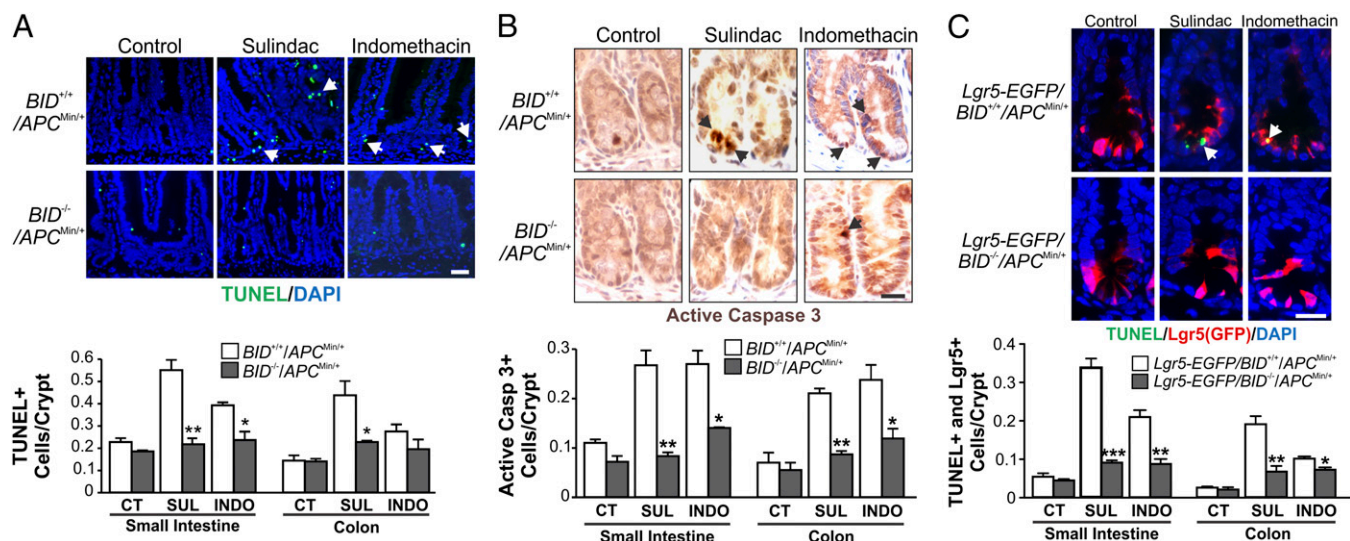


Fig. 3. BID deficiency abolishes NSAID-induced apoptosis in intestinal stem cells in $APC^{Min/+}$ mice. (A and B) $BID^{+/+}$ and $BID^{-/-}$ $APC^{Min/+}$ mice were fed control (CT) or experimental AIN93G diet containing 200 ppm sulindac (SUL) or 10 ppm indomethacin (INDO) for 1 wk. Small intestinal and colonic sections were analyzed for apoptosis by TUNEL staining (green) (A) and active caspase 3 staining (brown) (B). (Upper) Representative staining pictures of small intestinal sections, with arrows indicating example cells with positive staining. (Lower) Mean + SD of positive signals in the small intestinal and colonic crypts. (C) $Lgr5$ -EGFP-marked $BID^{+/+}$ and $BID^{-/-}$ $APC^{Min/+}$ mice were treated as in A and analyzed for intestinal stem cell apoptosis by TUNEL (green) and EGFP ($Lgr5$; red) double staining. (Upper) Representative staining pictures of small intestinal sections, with arrows indicating example double-positive cells. (Lower) Mean + SD of TUNEL/ $Lgr5$ double-positive signals in the small intestinal and colonic crypts. (Scale bars: 25 μ m.) * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ ($n = 3$ per group).

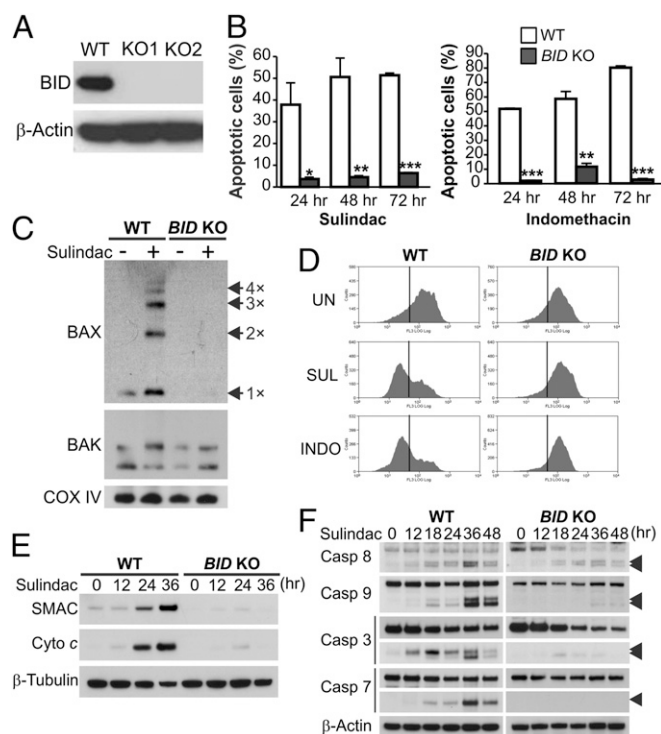


Fig. 4. BID mediates NSAID-induced apoptosis in CRC cells through the mitochondrial pathway. (A) Western blot of BID in WT and two independent clones of *BID* knockout (*BID* KO) HCT116 cell lines. (B) Apoptosis at indicated time points in WT and *BID* KO HCT116 cells treated with 120 μ M sulindac sulfide or 500 μ M indomethacin was analyzed by counting cells with condensed and fragmented nuclei. Results were expressed as means \pm SD of three independent experiments. * P < 0.05; ** P < 0.01; *** P < 0.001. (C) BAX and BAK multimerization was analyzed by cross-linking the mitochondrial fractions isolated from cells treated as in B for 36 h, followed by SDS/PAGE under nonreducing conditions and Western blotting. Cox IV, a mitochondrial protein, was used as a control for loading and fractionation. (D) Mitochondrial membrane potential in cells untreated (UN) or treated with sulindac sulfide (SUL) or indomethacin (INDO) as in B for 48 h was analyzed by MitoTracker Red staining followed by flow cytometry. (E) Cytochrome c (Cyto c) and SMAC/DIABLO release were analyzed by Western blotting of cytosolic fractions isolated from cells treated with sulindac sulfide as in C. α -Tubulin, a cytosolic protein, was used as control for loading and fractionation. (F) Western blot of caspase (Casp) activation at the indicated time points in cells treated as in B. Arrowheads indicate caspase cleavage fragments.

cleavage in NSAID-treated colon cancer cells (Fig. 5B and Fig. S6D). Similar to the finding in mice (Fig. S2C), caspase 8 activation was found in NSAID-treated *BID* KO cells (Fig. 4F), suggesting activation of BID through the extrinsic pathway. We therefore tested this possibility by perturbing the extrinsic pathway components. BID cleavage in sulindac-treated HCT116 cells was suppressed by transient knockdown of DR5 or stable knockdown of caspase 8 (Fig. 5C and D). Sulindac-induced apoptosis was blocked by stable knockdown of caspase 8 (Fig. 5D) and partially inhibited by transient knockdown of DR5 or expression of dominant negative FADD (Fig. 5C and Fig. S6E), which is likely due to incomplete inhibition of DR5 expression and FADD activity. Together, these results demonstrate that BID is activated by NSAIDs through the extrinsic pathway and mediates apoptosis induction by engaging the intrinsic pathway to amplify the apoptotic signal.

NSAID-Induced DR Signaling Triggers BID-Dependent Synthetic Lethality in APC-Deficient Cells with c-Myc Activation. The anticancer effect of NSAIDs depends on selective killing of neoplastic

cells (25). To determine this selectivity, we analyzed NCM356 normal intestinal epithelial cells (IECs), which have normal epithelial morphology and a functional APC pathway (26). NCM356 cells were resistant to NSAIDs and lacked BID cleavage, Bax multimerization, and caspases 8 and 3 activation, but remained sensitive to camptothecin and staurosporine (Fig. 5E and F and Fig. S6F). Surprisingly, DR5 induction and FLIP_s down-regulation were still observed in NCM356 cells following NSAID treatment (Fig. 5E). In both HCT116 and NCM356 cells, NSAID treatment similarly modulated the transcription and mRNA expression of the proximal DR pathway components, including *DR5*, *c-FLIP*, and the decoy receptors *Dcr1* and *Dcr3* (Fig. S7A–D). Lack of apoptotic response even with DR pathway modulation was verified in another colon-derived and normal cell line, CRL-1831 (Fig. S7E and F). Therefore, activation of the DR pathway by NSAIDs can occur in normal cells up to a point before caspase 8 and BID cleavage.

We then probed the selective activation of BID and caspase 8 by NSAIDs in neoplastic cells considering factors present in neoplastic cells but not normal cells. Loss of *APC* drives intestinal tumorigenesis by activating *c-Myc* (27, 28), which can

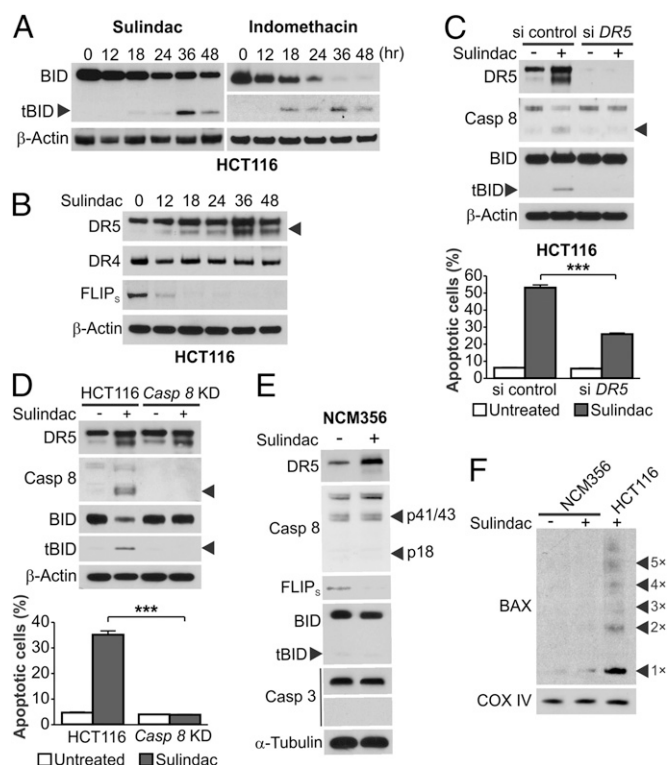


Fig. 5. BID is specifically activated by NSAIDs in cancer cells through the death receptor pathway. (A) Western blots of HCT116 cells treated with 120 μ M sulindac sulfide or 500 μ M indomethacin, with the arrowhead indicating tBID. (B) HCT116 cells were treated as in A, and analyzed for DR4, DR5, and FLIP_s expression at indicated time points by Western blotting. (C) HCT116 cells were transfected with control scrambled or DR5 siRNA, and then treated with sulindac sulfide as in A. (Upper) Western blot analysis of indicated proteins at 36 h after treatment. (Lower) Apoptosis was analyzed by counting condensed/fragmented nuclei after nuclear staining at 24 h after treatment. (D) Parental and stable caspase 8 knockdown (Casp 8 KD) HCT116 cells were treated with sulindac sulfide as in A. (Upper) Western blot analysis of indicated proteins at 24 h after treatment. (Lower) Apoptosis was analyzed as in C at 24 h after treatment. (E) Indicated proteins were analyzed by Western blotting in NCM356 cells treated with sulindac sulfide as in A for 36 h. (F) BAX multimerization in NCM356 and HCT116 cells was analyzed as in Fig. 4C. Results in C and D were expressed as means \pm SD of three independent experiments. *** P < 0.001.

of DR signaling and aberrant Wnt signaling, which can be caused by inherited or sporadic *APC* mutations, activating β -catenin mutations, or other alterations (2). NSAIDs do not discriminate between cancer and normal cells in modulating the expression of DR5, c-FLIP, and other DR family members, which is insufficient for cell death. However, these changes in conjunction with activation of Wnt signaling and ensuing c-Myc induction are both sufficient and required to trigger caspase 8 and BID cleavage, leading to apoptosis induction in an otherwise normal background. Therefore, premalignant cells with *APC* loss and aberrant Wnt signaling are primed for apoptosis due to c-Myc-induced DR signaling, and the enhanced DR signaling by NSAIDs helps reach a critical threshold for caspase 8 activation (Fig. 6F). NSAIDs induce a number of apical signaling events (21), some of which, such as endoplasmic reticulum (ER) stress, can trigger DR signaling (35). Studying these signaling events may help identify the direct target of NSAIDs in chemoprevention, which should be an important area of future investigation. Exploiting synthetic lethal interactions has been recognized as a powerful targeting strategy (36) and may hold the key for developing effective preventive agents for other cancer types with defined gatekeeper or driver mutations.

Materials and Methods

Analysis of Human Colonic Polypos. Frozen specimens from patients with sporadic colonic adenomas, including nine patients treated with NSAIDs and seven untreated patients, were acquired from the Health Sciences Tissue Bank of the University of Pittsburgh. Acquisition of the tissue samples was approved by the Institutional Review Board. Informed consent was received from all participating patients. TUNEL and the intestinal stem cell marker OLFM4 were analyzed by immunostaining of paraffin-embedded tissue as described (16). Active caspase 8 staining was performed as described in *SI Materials and Methods*.

Mice and Treatment. The procedures for all animal experiments were approved by the Institutional Animal Care and Use Committee of the University of Pittsburgh. C57BL/6J *BID*^{-/-} (*BID* KO) mice, which were described (19), were crossed with *APC*^{Min/+} mice (Jackson Laboratory) to generate *APC*^{Min/+} mice with different *BID* genotypes. *Lgr5-EGFP*-marked *BID*^{+/+} and *BID*^{-/-} *APC*^{Min/+} mice were generated as described in *SI Materials and Methods*. For NSAID treatment, 4-wk-old mice were fed AIN-93G diets (Dyets) with or without 200 ppm sulindac (Sigma) or 10 ppm indomethacin (Sigma) for 1 wk for apoptosis analysis, or for 2 or 4 mo for tumor phenotype analysis. Following treatment and killing of mice, analysis of adenoma numbers and apoptosis in adenoma tissues was done as described in *SI Materials and Methods*. For survival analysis, *BID*^{+/+} and *BID*^{-/-} *APC*^{Min/+} mice were fed sulindac or control diet for up to 70 wk when all mice were killed.

Analysis of NSAID-Induced and BID-Mediated Apoptosis in Human CRC Cell Lines and NCM356 Cells. Human CRC cell lines, including HCT116, HT29, RKO, DLD1 and their derivatives, and NCM356 cells (Incell) (26), were treated by NSAIDs and analyzed for apoptosis by nuclear staining, annexin V/propidium iodide staining, colony formation, mitochondrial membrane integrity, cytochrome c release, and Bax conformational change as described in *SI Materials and Methods*.

Statistical Analysis. Statistical analyses were performed by using GraphPad Prism IV software. Differences were considered significant if the probability of the difference occurring by chance was less than 5 in 100 ($P < 0.05$).

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